Acetate represents a major product of heptanoate and octanoate β-oxidation in hepatocytes isolated from neonatal piglets

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An experiment was conducted to explore the nature of the radiolabel distribution in acid-soluble products (ASPs) resulting from the oxidation of [1-14C]C7o or C8o by isolated piglet hepatocytes. The differences between odd and even chain-length and the impacts of valproate and malonate upon the rate of β-oxidation and ASP characteristics were tested. A minor amount of fatty acid carboxyl carbon (<10% of organic acids identified by radio-HPLC) accumulated in ketone bodies regardless of chain-length or inhibitor used. In all cases, acetate represented the major reservoir of carboxyl carbon, accounting for 60–70% of radiolabel in identified organic acids. Cells given [1-14C]C7o accumulated 85% more carboxyl carbon in Krebs cycle intermediates when compared with C8o, while accumulation in acetate was unaffected. The results are consistent with the hypothesis that anapleros from odd-carbon fatty acids affects the oxidative fate of fatty acid carbon. The piglet appears unique in that non-ketogenic routes of fatty acid carbon flow (i.e. acetogenesis) predominate in the liver of this species.

INTRODUCTION

Ketone bodies, i.e., β-hydroxybutyrate and acetoacetate, are considered to be the predominant end-products of accelerated hepatic mitochondrial β-oxidation of fatty acids. Studies with numerous animal models have shown that under conditions of fasting [1], diabetes [2], medium-chain-length triacylglycerol administration [3–5], and during suckling [6,7], fatty acid oxidation is brisk, and levels of ketone bodies in the blood rise concomitant with a high ketogenic rate. Based on data from adult fasted rats [8], it has been presumed that ketone bodies comprise the majority of the non-CO2 carbon derived from oxidation of fatty acids with incubations of hepatocytes from piglets [9,10]. While this assumption may be true for species possessing significant hepatic ketogenic capacity, it may be questioned in animals whose capacity is low.

Neonatal hyperketonaemia is not characteristic of suckling piglets [11,12], suggesting that the rate of ketogenesis is minimal in newborn pigs relative to other neonates. Indeed, medium-chain fatty acid (MCFA: 6–12 carbons) administration to 24-h-old fasted piglets failed to increase the plasma β-hydroxybutyrate concentration above 40–60 µM [10,13,14], even though similar treatments induce marked ketonemia in dogs, humans and rats [3–5]. The rate of acid-soluble product (ASP) generation from hepatocytes incubated with [1-14C]octanoate or [1-14C]oleate in piglets is 57% lower [15,16] than in neonatal rabbits [17,18], and is a fraction of that observed in preparations from fasted adult rats [19].

Accumulation of carboxyl carbon in ASP from piglet hepatocyte incubations is far greater (by 45%) for cells given odd-chain fatty acids (C7o and C8o) as substrate [15]; these fatty acids were expected to provide anaplerotic carbon to the Krebs cycle and thereby diminish ketogenesis. This prompted Odle et al. [10] to suggest that ASP from poorly ketogenic piglet hepatocytes may include organic acids other than ketone bodies, and that chain-length differences may exist with respect to the organic acid profile in the ASP. Herein, we examined these problems by measuring β-oxidation and metabolic end-products of fatty acid oxidation in hepatocytes isolated from colostrum-deprived piglets. Attempts were made to alter carbon flow via inhibition of β-oxidation (inclusion of valproate) or blockage of the Krebs cycle (addition of malonate). The potential for carnitine to ameliorate the inhibitory effects of valproate on fatty acid metabolism [20–22] and to maintain cellular CoA status was addressed. HPLC/radiochromatographic characterization of radiolabelled organic acids resulting from MCFA β-oxidation led to the novel finding that the majority (i.e. >70%) of identifiable carboxyl-carbon accumulation occurred in acetate. These results are consistent with our previous description of major radioactivity residing in an HPLC peak believed to represent acetate in ASP from 1-14C-labelled fatty acid incubations with hepatocytes derived from 24-h-old newborn piglets [23].

MATERIALS AND METHODS

Animals and hepatocyte isolation

Yorkshire/Duroc piglets (n = 5, mass 811 ± 50 g) were obtained at farrowing and deprived of colostrum until hepatocyte isolation was carried out 24 h post partum. After animals were anaesthetized with pentobarbital (20 mg/kg), livers were cannulated, perfused with collagenase in retrograde fashion, and cells isolated as described previously [10]. Yields ranged from 1.2 × 10⁶ to 2.3 × 10⁶ hepatocytes/liver. Hepatocyte viability (Trypan Blue exclusion) was routinely >95% using this method.

Hepatocyte incubations

The hepatocyte incubations with 1-14C-labelled fatty acids were conducted as described previously [10]. Hepatocytes [8–15 × 10⁶ cells] were incubated in 3 ml of Krebs–Henseleit buffer (pH 7.4) at 37°C. For those flasks in which radioactivity in CO2 and ASP was of interest, incubations were terminated when 500 µl of 30% (w/v) trichloroacetic acid was injected into the medium

Abbreviations used: MCFA, medium-chain fatty acid; ASP, acid-soluble product.

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at 60 min. The contents of the flask were transferred to test tubes, centrifuged at 1200 \( g \) for 10 min, and the supernatant extracted six times with water-saturated hexane. Radioactivity in ethanolamine in centre wells (containing trapped \(^{14}\)CO\(_2\)) and in 150 \( \mu l \) aliquots of the acid-soluble fraction was determined using a scintillation counter after mixing with 10 ml of Biosafe II cocktail (Research Products International, Mt. Prospect, IL, U.S.A.). All radioactivity data were corrected for radioactivity in the blank (water-saturated hexane). When CoA and CoA esters were to be assayed, incubations using non-radioactive substrate were incubated in the presence or absence of valproate (1.5 mM) or malonate (10 mM), alone, or in combination with \( l \)-carnitine (3 mM), resulting in a total of 12 treatments. Valproate flasks were preincubated for 10 min at 37 °C prior to fatty acid addition. The accumulation of substrate [\(^{1-14}\)C]carboxyl carbon in \( \text{CO}_2 \), ASP and organic acids across treatments was monitored with addition of a tracer amount of [\(^{13}\)C]MCFA (1.6 and 1.8 \( \mu Ci/\mu mol \) for \( C_{16:0} \) and \( C_{18:0} \), respectively) in one set of flasks. A selected number of flasks were incubated for 20 min with non-radioactive MCFA/ \( l \)-carnitine (control), MCFA/\( l \)-carnitine/malonate, and MCFA/\( l \)-carnitine/valproate. For these samples, \( \text{O}_2 \) consumption was then measured polarographically using water-jacketed (37 °C) Clark electrodes coupled to a YSI Model 5300 \( \text{O}_2 \) monitor (Yellow Springs, OH, U.S.A.). The xanthine oxidase method was used for electrode calibration [24].

### Organic acids

An aliquot of the acid-soluble fraction was subjected to ion-exchange HPLC/radiochromatographic analysis for organic acids as described previously [23] and modified as follows: flow rate was 0.65 ml/min and the ratio of \( \beta \)-flowmonitor cocktail and HPLC eluent was 5:1 (v/v). This method resolves organic acids into nine UV peaks, except that acetate co-elutes with acetoacetate (Figure 1) [23]. Radioactivity accumulated in organic acids was determined after collection of eluent into vials at appropriate times (see Figure 1) using a fraction collector (Isco Retriever II, Lincoln, NE, U.S.A.). The retention times of a number of acids were too close to allow for separate collection. In these cases (see Table 1), eluent was collected across the proximate organic acids (i.e. fraction A in Figure 1) and their radioactivity summed. For some samples (see the Results section), reversed-phase ion-pairing HPLC radiochromatography was used to characterize radioactivity accumulated in acetate and ketone bodies (Figure 2). This system employed a Beckman...

### Table 1: Accumulation of carboxyl carbon in \( \text{CO}_2 \) and ASPs from \( 1-14\)-labelled \( C_{16:0} \) and \( C_{18:0} \) plus carnitine and/or inhibitors incubated with hepatocytes isolated from 24-h-old colostrum-deprived piglets

Incubation conditions are given in the text (\( n = 5 \) piglets throughout Table). \( * P < 0.0001 \), significant difference between \( C_{16:0} \) versus \( C_{18:0} \). Means with different superscripts within a column are significantly different (\( P < 0.01 \) for \( \text{CO}_2 \) and \( P < 0.0001 \) for ASP).

<table>
<thead>
<tr>
<th>Treatment/additions</th>
<th>( \text{CO}_2 )</th>
<th>ASP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( C_{16:0} )</td>
<td>( C_{18:0} )</td>
</tr>
<tr>
<td>Substrate</td>
<td>13.1 ± 3.4(^a)</td>
<td>8.6 ± 2.1(^a)</td>
</tr>
<tr>
<td>+ Carnitine</td>
<td>12.5 ± 3.2(^a)</td>
<td>8.2 ± 2.1(^a)</td>
</tr>
<tr>
<td>+ Valproate</td>
<td>11.1 ± 2.9(^a)</td>
<td>7.7 ± 1.9(^a)</td>
</tr>
<tr>
<td>+ Carnitine and valproate</td>
<td>11.1 ± 2.5(^a)</td>
<td>6.7 ± 1.8(^a)</td>
</tr>
<tr>
<td>+ Malonate</td>
<td>12.7 ± 2.5(_{0.05})</td>
<td>8.0 ± 2.0(_{0.05})</td>
</tr>
<tr>
<td>+ Carnitine and malonate</td>
<td>11.9 ± 2.9(_{0.05})</td>
<td>7.9 ± 2.3(_{0.05})</td>
</tr>
</tbody>
</table>
Initial 2.5 min of each run, eluent consisted of 99% $A$ over 2.5 min, 10% $B$ over 8 min, and 100% $B$ over 40 min. The retention time of key compounds was maintained for 28.5 min and then increased to 1.2 ml/min over 15 min. CoA and acetyl-CoA in the cell pellet extracts were readily measured; however, other CoA esters could not be quantified using this method.

**Chemicals**

$[1-^{14}C]$Heptanoate (3.2 µCi/µmol) and $[1-^{14}C]$octanoate (55 µCi/µmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). Carnitine was a gift from Lonza (Basel, Switzerland). Ethanolamine was from Eastman Kodak (Rochester, NY, U.S.A.). All other chemicals, including organic acid salts, CoA and its derivatives, sodium valproate, and sodium malonate, were purchased from Sigma (St. Louis, MO, U.S.A.).

**Statistics**

Data were analysed statistically using the general linear models procedure of SAS (SAS Institute, Cary, NC, U.S.A.), considering the effects of fatty acid substrate ($C_{7}\alpha$ versus $C_{8}\alpha$), carnitine, inhibitor (valproate or malonate), and interactions thereof. Differences in means for main effects or interactions were compared when found to be significant ($P < 0.05$) in the analysis of variance. The means ± S.E.M. are reported unless otherwise noted. Statistical interactions between fatty acid–carnitine, carnitine–inhibitor, or fatty acid–carnitine–inhibitor were not significant for any variable tested ($P > 0.1$), and for brevity are not reported in the text or Figure/Table legends.

**RESULTS**

**Accumulation of label in CO$_2$ and ASP**

The accumulation of the carboxyl carbon of $C_{7}\alpha$ or $C_{8}\alpha$ in CO$_2$ was affected significantly by fatty acid chain-length and inhibitor used (Table 1). Across treatments, accumulation from $C_{7}\alpha$ was 54% greater than from $C_{8}\alpha$. Compared with substrate alone, there was no effect of malonate, and a 14% depression with valproate. There was no effect of carnitine across substrates or inhibitors ($P > 0.1$).

Carboxyl-carbon accumulation in ASP was also significantly altered by substrate chain-length and inhibitor. Accumulation was 46% higher with $C_{7}\alpha$ versus $C_{8}\alpha$. Valproate-inhibited cells accumulated carboxyl carbon in ASP to only 55% of that observed in controls or in cells incubated with malonate. No significant effects of malonate or carnitine were observed ($P > 0.1$).

Addition of inhibitor had a significant ($P < 0.0001$) impact on the pattern of carboxyl radiolabel partitioning between CO$_2$ and ASP. Relative accumulation in ASP, expressed as a percentage of total radiolabel accumulation in ASP + CO$_2$ (i.e., % of total oxidative flux), was lower in cells incubated with valproate (49%) when compared with controls (59%) or those incubated with malonate (62%; S.E.M. = 0.8%). The difference ($P = 0.02$) in relative ASP accumulation between $C_{7}\alpha$ (56%) and $C_{8}\alpha$ (58%) was small (S.E.M. = 0.6%). Across all treatments, the percentage of total accumulation of radiolabel into ASP ranged from 46 to 62% (results not shown). Again, no effect of carnitine was observed ($P > 0.1$).

**Oxygen consumption**

Oxygen consumption was not affected by inclusion of valproate or malonate in the medium (results not shown). However, fatty acid chain-length affected ($P < 0.0001$) the rate of $O_2$ utilization across treatments, being 7% greater for $C_{7}\alpha$ (323 nmol of $O_2$/h per 10$^6$ cells) than $C_{8}\alpha$ (301 nmol of $O_2$/h per 10$^6$ cells).
Table 2  Free CoA and acetyl-CoA concentrations in hepatocytes isolated from 24-h-old colostrum-deprived piglets before incubation (blank) and after incubation with non-radioactive C7:0 or C8:0 substrate in the absence or presence of carnitine and/or inhibitors

<table>
<thead>
<tr>
<th>Treatment/additions</th>
<th>Free CoA (nmol/g wet wt of cells)</th>
<th>Acetyl-CoA (nmol/g wet wt of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C7:0</td>
<td>C8:0</td>
</tr>
<tr>
<td>Blank</td>
<td>15.2 ± 4.3</td>
<td>12.6 ± 4.1</td>
</tr>
<tr>
<td>Substrate</td>
<td>6.6 ± 3.4</td>
<td>6.6 ± 1.7</td>
</tr>
<tr>
<td>+ Carnitine</td>
<td>7.3 ± 3.4</td>
<td>6.2 ± 2.4</td>
</tr>
<tr>
<td>+ Valproate</td>
<td>3.9 ± 2.1</td>
<td>4.6 ± 2.2</td>
</tr>
<tr>
<td>+ Carnitine and valproate</td>
<td>5.0 ± 2.4</td>
<td>4.9 ± 2.1</td>
</tr>
<tr>
<td>+ Malonate</td>
<td>8.9 ± 2.8</td>
<td>5.7 ± 2.1</td>
</tr>
<tr>
<td>+ Carnitine and malonate</td>
<td>6.2 ± 3.3</td>
<td>6.0 ± 2.1</td>
</tr>
</tbody>
</table>

Table 3  Carboxyl carbon accumulation in organic acids derived from the ASP fraction of newborn piglet hepatocyte incubations

Cells were incubated with [1-14C]-citrate or [1-14C]-acetate, and the organic acids separated by ion-exchange HPLC. Values in the far right column represent the percentage of total ASP radioactivity which could be accounted for in the metabolites listed, and is calculated thus: (summed d.p.m. in all identified metabolite peaks) / (total ASP d.p.m.) × 100. Acetate values represent a combination of acetate + acetoacetate accumulation. However, accumulation in acetoacetate was negligible (see the text and Table 4).

<table>
<thead>
<tr>
<th>Carboxyl carbon accumulation (natom/h per 10^6 hepatocytes)</th>
<th>Treatment/additions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate/α-oxoglutarate/</td>
<td>Pyruvate/ malate</td>
</tr>
<tr>
<td>oxaloacetate</td>
<td></td>
</tr>
<tr>
<td>C7:0 substrate</td>
<td>0.68 ± 0.09†</td>
</tr>
<tr>
<td>+ Carnitine</td>
<td>0.72 ± 0.08</td>
</tr>
<tr>
<td>+ Valproate</td>
<td>0.33 ± 0.03</td>
</tr>
<tr>
<td>+ Carnitine and valproate</td>
<td>0.47 ± 0.10</td>
</tr>
<tr>
<td>+ Malonate</td>
<td>0.69 ± 0.12</td>
</tr>
<tr>
<td>+ Carnitine and malonate</td>
<td>0.66 ± 0.09</td>
</tr>
<tr>
<td>C8:0 substrate</td>
<td>0.27 ± 0.05</td>
</tr>
<tr>
<td>+ Carnitine</td>
<td>0.29 ± 0.04</td>
</tr>
<tr>
<td>+ Valproate</td>
<td>0.18 ± 0.05</td>
</tr>
<tr>
<td>+ Carnitine and valproate</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>+ Malonate</td>
<td>0.28 ± 0.05</td>
</tr>
<tr>
<td>+ Carnitine and malonate</td>
<td>0.30 ± 0.04</td>
</tr>
</tbody>
</table>

* Significant difference between C7:0 and C8:0 across treatments (*P < 0.01).† Significant effect of valproate/malate inhibitors (**P < 0.01).‡ Significant interaction of fatty acid and inhibitor (**P < 0.05).

CoA and acetyl-CoA levels

Across fatty acid substrates, the treatments resulted in a substantial 52% (substrate alone or malonate) to 67% (valproate) drop in CoA levels relative to the 13.9 nmol/g wet wt of cells measured in time-zero blanks (Table 2). The drop in CoA was unaffected by carnitine or chain-length of fatty acid.

The slight decrease in acetyl-CoA concentration with the addition of carnitine was not statistically significant. The difference between C7:0 and C8:0 was significant (Table 2). Acetyl-CoA levels across treatments were just 55% (substrate only), 64% (malonate), and 45% (valproate) of that determined for blanks (24.3 nmol/g wet wt of cells).

HPLC/radiochromatography of organic acids

The radioactivity accumulated in the organic acid peaks resolved by ion-exchange HPLC/radiochromatography is presented in Table 3. Of the organic acids identified, which included the ketone bodies, accumulation of carboxyl carbon in acetate was predominant across all treatments. Relative to acetate, the contribution of acetoacetate to the carboxyl accumulation in the acetate/acetoacetate HPLC fraction was presumed to be small because (a) accumulation in β-hydroxybutyrate was a small fraction of identifiable organic acid accumulation (Table 3), (b) ketogenesis from in vitro piglet liver preparations incubated with fatty acids has been shown to be low [16,19], and (c) in vivo assessments of ketogenic capacity in piglets have repeatedly underscored their low ketogenic potential [i.e. [10,13,14]]. Nevertheless, it was necessary to develop a method which resolved these metabolites in order to quantify radioactivity in acetoacetate. Reversed-phase ion-pairing HPLC was thus employed on ASP samples from two replicates. As judged by elution of radioactive compounds through in-line radiochromatography (Figure 2), and by quantification of radioactivity associated with eluent corresponding to these metabolites (Table 4), only minor amounts of carboxyl carbon accumulated in acetoacetate or β-hydroxybutyrate relative to acetate. Across treatments or fatty acid chain-length, acetoacetate accounted for a maximum of only 3% of ASP radioactivity (6% of identifiable...
organics (citrate, fatty acids, etc.) as a reservoir of carboxyl carbon. The finding that acetogenesis far surpasses ketogenesis in piglet hepatocytes appears unique. Nevertheless, substantial endogenous (non-fermentative) production of acetate has been described in rat and sheep liver [26,27]. Plasma acetate doubled in humans fasted for 4.5 days [28], and is elevated in diabetic humans, rats and sheep [26,27,29], suggesting that endogenous acetogenesis occurs concomitant with ketogenesis. β-Oxidative end-products not arising from ketogenesis (i.e. acetyl-carnitine) have been described in rat muscle and liver under various conditions [30,31].

It is intriguing that when ASP samples were subjected to reversed-phase ion-pairing HPLC, a large fraction (up to 70%) of radiolabel accumulated in a peak as yet unidentified (Figure 2). It is notable that the percentage of unidentified ASP radioactivity was significantly affected by differences in fatty acid and by the presence of valproate (see Tables 3 and 4), supporting the view that this fraction is related to cellular metabolism. The unidentified fraction could, in theory, represent such metabolites as acetyl-carnitine, glucose, glutamate, etc. Clarification of this phenomenon will be required.

**Carboxyl-carbon accumulation in CO₂ and ASP**

Odle et al. [10] reported a higher (by 45%) accumulation of carboxyl carbon in oxidative flux from cells given odd-chain MCFA. Consistent with this finding, we observed that C₇₀ significantly increased radioactivity accumulation in both CO₂ and ASP relative to C₈₀ (Table 1). Furthermore, results from radio-HPLC analysis established that the carboxyl-carbon accumulation in Krebs cycle intermediates was 85% higher in cells incubated with C₇₀ versus C₈₀ while flux to β-hydroxybutyrate increased by a mere 12% (Table 3) and accumulation in acetate was unaffected. These results support the hypothesis that anaplerosis from odd-chain MCFA expands the pool of Krebs cycle intermediates, thereby resulting in more complete com-

**Table 4 Distribution of ASP radioactivity in acetocacetate, acetate and unidentified compound(s) from hepatocytes isolated from 24-h-old colostrum-deprived piglets and incubated with [1-¹³C]C₇₀ or [1-¹³C]C₈₀ in the presence or absence of carnitine and/or inhibitors**

Alloquots of ASP across all treatments from two replicates were subjected to reversed-phase ion-pairing HPLC (see the Materials and methods section), and peaks eluting with retention times corresponding to acetocacetate, acetate and an unidentified radioactive entity (Table 2) were collected and counted for radioactivity. Radioactivity in each peak was corrected for d.p.m. determined at equivalent retention times from ASP blanks (acid-killed at time 0 incubation). *P < 0.0003; significant difference between C₇₀ and C₈₀. Means with different superscripts within a column are significantly different (P < 0.05).

<table>
<thead>
<tr>
<th>Treatment/additions</th>
<th>Unknown</th>
<th>Acetate</th>
<th>Acetoacetate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C₇₀</td>
<td>C₈₀</td>
<td>C₇₀</td>
</tr>
<tr>
<td>Substrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Carnitine</td>
<td>52.1 ± 1.4a</td>
<td>41.2 ± 6.9a</td>
<td>32.3 ± 2.4a</td>
</tr>
<tr>
<td>+ Valproate</td>
<td>50.4 ± 4.3a</td>
<td>33.8 ± 3.0a</td>
<td>38.7 ± 0.8a</td>
</tr>
<tr>
<td>+ Carnitine and valproate</td>
<td>76.0 ± 11.8b</td>
<td>47.2 ± 5.1b</td>
<td>19.3 ± 1.6b</td>
</tr>
<tr>
<td>+ Malonate</td>
<td>72.6 ± 4.9c</td>
<td>43.5 ± 3.2c</td>
<td>26.5 ± 0.3c</td>
</tr>
<tr>
<td>+ Carnitine and malonate</td>
<td>56.5 ± 7.2</td>
<td>36.4 ± 5.1</td>
<td>35.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>53.5 ± 5.7d</td>
<td>32.2 ± 3.8d</td>
<td>38.0 ± 0.1d</td>
</tr>
</tbody>
</table>

**Acetogenesis from fatty acids in piglet liver**

**Organic acid profile**

Numerous studies [11–14,19] have illustrated that neonatal pigs display a minimal ketogenic capacity. These studies prompted the development of an ion-exchange HPLC/radiochromatographic method for analysis of organic acids in ASP from piglet hepatocytes incubated with 1-¹³C-labelled fatty acids which showed that major carboxyl carbon radioactivity was in a peak corresponding to acetate/acetoacetate [23]. However, radioactivity in other organic acids was not obtained, and the complete characterization of β-oxidative end-products derived from piglet hepatocytes remained unclear. The current study extends the previous experiment [23], showing major accumulation of radioactivity in acetate and an unidentified entity (Table 4). Under all conditions studied the ketone bodies were a minor reservoir of carboxyl carbon. The finding that acetogenesis far surpasses ketogenesis in piglet hepatocytes appears unique. Nevertheless, substantial endogenous (non-fermentative) production of acetate has been described in rat and sheep liver [26,27]. Plasma acetate doubled in humans fasted for 4.5 days [28], and is elevated in diabetic humans, rats and sheep [26,27,29], suggesting that endogenous acetogenesis occurs concomitant with ketogenesis. β-Oxidative end-products not arising from ketogenesis (i.e. acetyl-carnitine) have been described in rat muscle and liver under various conditions [30,31].

It is intriguing that when ASP samples were subjected to reversed-phase ion-pairing HPLC, a large fraction (up to 70%) of radiolabel accumulated in a peak as yet unidentified (Figure 2). It is notable that the percentage of unidentified ASP radioactivity was significantly affected by differences in fatty acid and by the presence of valproate (see Tables 3 and 4), supporting the view that this fraction is related to cellular metabolism. The unidentified fraction could, in theory, represent such metabolites as acetyl-carnitine, glucose, glutamate, etc. Clarification of this phenomenon will be required.

**DISCUSSION**

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Odle et al. [10] reported a higher (by 45%) accumulation of carboxyl carbon in oxidative flux from cells given odd-chain MCFA. Consistent with this finding, we observed that C₇₀ significantly increased radioactivity accumulation in both CO₂ and ASP relative to C₈₀ (Table 1). Furthermore, results from radio-HPLC analysis established that the carboxyl-carbon accumulation in Krebs cycle intermediates was 85% higher in cells incubated with C₇₀ versus C₈₀ while flux to β-hydroxybutyrate increased by a mere 12% (Table 3) and accumulation in acetate was unaffected. These results support the hypothesis that anaplerosis from odd-chain MCFA expands the pool of Krebs cycle intermediates, thereby resulting in more complete com-
bustion of acetyl-CoA units to CO₂ with less acetyl-CoA flux into ketone bodies or acetate.

The capability of valproate to lower β-oxidative flux and ketogenesis has been documented repeatedly in vivo [22,32] and in vitro [20,21], and is thought to be due to a combination of competition with fatty acid substrate for a limited mitochondrial CoA pool [20,22,31], and to some degree an effect upon β-oxidative enzymes [33]. Indeed, CoA was lowered by 32% in cells incubated with valproate versus those incubated with substrate alone or with malonate (Table 2), consistent with CoA sequestration by valproate or its metabolites. There is evidence that carnitine may help to relieve some negative effects of valproate in vivo, presumably through formation of acyl-carnitine esters which may liberate CoA. In support of this notion, carnitine was found to raise C₈₆₀ oxidation to CO₂ by approx. 7% in vivo, and depressed C₅₀₉₀- and C₇₀₉₀-oxidation of dicarboxylic acid excretion by approx. 50% in animals given C₈₀₆₀+valproate [35]. Thus, the precise role of carnitine in alleviating the negative effects of valproate upon β-oxidation is not entirely clear.

Inclusion of malonate at 10 mM failed to affect carboxyl-carbon accumulation in ASP, CO₂, or β-hydroxybutyrate. Evidence of succinate dehydrogenase inhibition by malonate was obtained (Table 3), in that addition of malonate increased accumulation in succinate by 3.6- and 1.5-fold (C₅₀₆₀ and C₇₀₆₀, respectively), and depressed accumulation in pyruvate/malate by 25% (C₅₀₆₀ only) and in fumarate by 39% (C₇₀₆₀ only); however, the Krebs cycle organic acids were a minor pool of carboxyl carbon. The lack of strong malonate effects upon total ASP and CO₂ in this study might be related to the use of hepatocytes rather than mitochondria.

Free CoA and acetyl-CoA

The oxidation of fatty acids, which requires free CoA, should theoretically increase the level of acetyl-CoA and lower the content of free CoA in hepatocytes. Consistent with this, neonatal piglet hepatocytes incubated with MCFA displayed a free CoA content much lower than that in blank preparations (Table 2), and liver acetyl-CoA concentration increased with gastric administration of long-chain fatty acids in piglets [36]. Valproate further lowered the free CoA concentration, probably via formation of valproyl- and other acyl-CoA esters. However, with addition of 1 mM MCFA, cellular acetyl-CoA content was diminished relative to blanks (Table 2). Qualitatively similar results were obtained by Kempen and Odle [35], who observed a drop in liver acetyl-CoA and free CoA after in vivo infusion of C₈₀₆₀ in neonatal piglets. It is constructive to emphasize that the concentration of acetyl-CoA was extremely low from neonatal piglets (this study and [35,36]) compared with rats [37]. Explanations for this finding are only speculative, but it is possible that (1) in the presence of MCFA, CoA becomes bound in other acyl-CoA moieties (i.e. butyryl- and hexanoyl-CoA for cells given C₈₀₆₀) which were not quantifiable with the HPLC analysis employed, and (2) piglets fail to accumulate acetyl-CoA which could be related to a high concentration of acetate generated from acetyl-CoA in the cells. If so, a high activity of acetyl-CoA hydrolase in pig hepatocytes may be expected.

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